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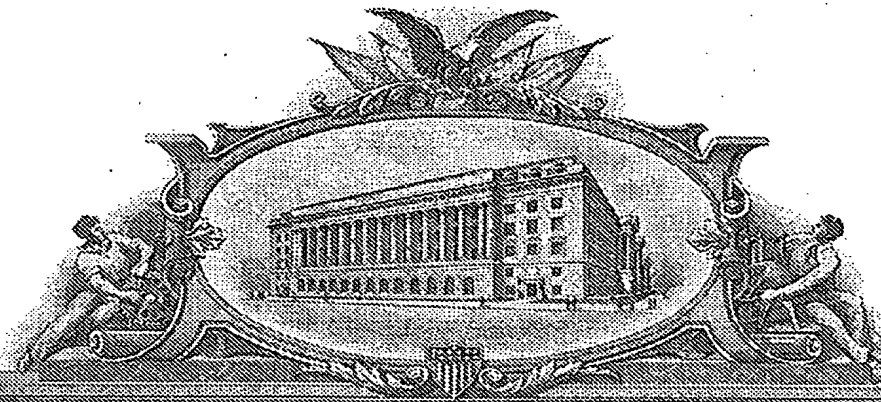
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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INVENTOR(S)					
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Susann		Taudte		Pennsburg, PA	
<input type="checkbox"/> Additional inventors are being named on the separately numbered sheets attached hereto					
TITLE OF THE INVENTION (500 characters max)					
PURIFICATION OF GLYCOPEGYLATED PEPTIDES USING HYDROPHOBIC INTERACTION CHROMATOGRAPHY (HIC)					
Direct all correspondence to: CORRESPONDENCE ADDRESS					
<input checked="" type="checkbox"/> Customer Number		043850			
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ENCLOSED APPLICATION PARTS (check all that apply)					
<input type="checkbox"/> Specification Number of Pages		24		<input type="checkbox"/> CD(s), Number	
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METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT					
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27.					
<input type="checkbox"/> A check or money order is enclosed to cover the filing fees					
<input checked="" type="checkbox"/> The Director is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 50-0310					
Payment by credit card. Form PTO-2038 is attached.					
FILING FEE Amount (\$) 100.00					
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.					
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[Page 1 of 2]

Respectfully submitted,

Date 01/10/05

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

PURIFICATION OF GLYCOPEGYLATED PEPTIDES USING HYDROPHOBIC INTERACTION CHROMATOGRAPHY (HIC)

Summary of the Invention

The present invention provides a route to purify PEG-GCSF, e.g., glyco-pegylated GCSF, that makes use of HIC as a second purification step, which is used in addition to cation exchange to remove contaminants other than un-pegylated GCSF.

In an exemplary embodiment, the invention provides a method of purifying glyco-pegylated GCSF, that has been passed through an initial purification on a gel permeation chromatography column, such as a SPHP column.

MATERIALS AND METHODS

Materials

- PEG-GCSF reaction mixtures (variety of lots)
- SP Sepharose High Performance – SPHP (Amersham)
- Phenyl Sepharose 6 Fast Flow (high sub) – PSFF (high sub, Amersham)
- Butyl Sepharose 4 Fast Flow – BSFF (Amersham)
- Butyl Toyopearl 650M – B650M (TosoHaas)
- Phenyl Toyopearl 650M – P650M (TosoHaas)
- TSKgel G3000SW_{XL} (TOSOH Biosciences) and Shodex OHpak column (Phenomenex)

Methods

All procedures were performed at room temperature (22-28°C).

1. SPHP Purification of PEG-GCSF. A PEG-GCSF reaction mixture was diluted appropriately and applied onto a pre-equilibrated SP Sepharose High Performance column. The column was washed with low salt buffer (20-50mM NaOAc/ 5mM NaCl pH 3-4.5) or 95% low salt buffer/ 5% high salt buffer (20-50mM NaOAc/ 1M NaCl pH 3-4.5). Protein was eluted using a gradient from low to high salt buffer. The column was regenerated with 0.5M NaOH.

2. HIC resin screen. SPHP purified PEG-GCSF was adjusted to 20mM NaOAc pH 4.5 and the desired NaCl concentration using stock solutions. Salted-up solutions were applied onto drip columns containing approximately 0.5ml of Phenyl SFF, Butyl SFF, Butyl 650M, or Phenyl 650M. The flow through was collected, columns were washed with a wash buffer containing the same NaCl and NaOAc concentrations as the load. Protein was eluted with 20mM NaOAc pH 4.5 followed by an H₂O wash and regeneration with 0.5M NaOH. 30µl of each sample including load, flow through, wash, NaOAc, H₂O elutions and regen were loaded on a 4-20% Tris-glycine gel.

3. HIC Purification of PEG-GCSF. SPHP purified PEG-GCSF or SPHP purified PEG-GCSF combined with SPHP purified nonpegylated GCSF was adjusted to 10-20mM NaOAc pH 4-4.5 and the desired salt concentration using stock solutions. The salted-up solution was loaded onto a pre-equilibrated column (1ml HiTrap Phenyl FF high sub or 1ml Phenyl Toyopearl 650M), washed with a 20mM NaOAc pH 4-4.5 buffer containing the same salt concentration as the load, and eluted (using either step or gradient elution) with H₂O or 20mM NaOAc pH 4-4.5.

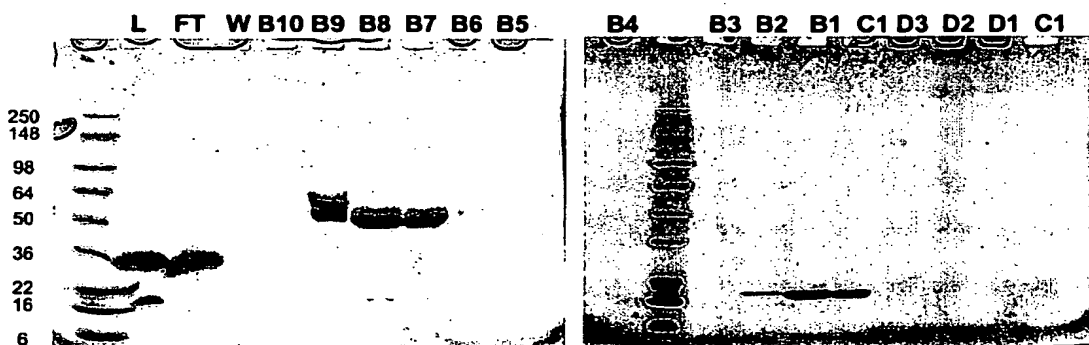
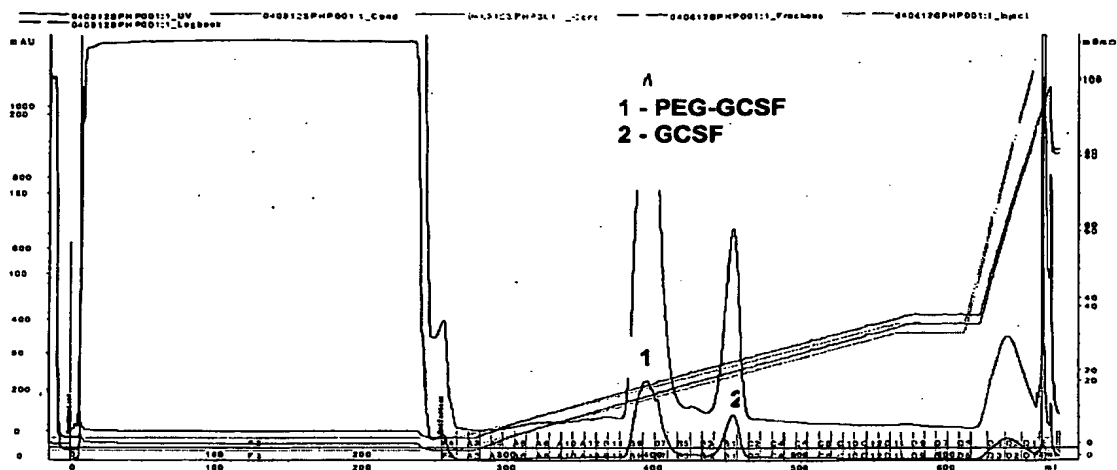
4. SEC analysis. SEC analysis was performed using a TSKgel G3000SW_{XL} (TOSOH Biosciences, 7.8mmIDx30cm, 5 μ m, Cat# 08541) and a Shodex OHpak column (Phenomenex, 8mmIDx30cm, Cat# SB-804HQ). 20% (v/v) of 50mM NaOAc/ 250mg/ml sorbitol/ 0.004% Tween 80 was added to each sample and allowed to adjust to room temperature prior to loading onto a G3000SW_{XL} column. Analysis on a Shodex OHpak column was performed. Samples for analyses on a Shodex OHpak column were diluted 2-fold with a 0.008% Tween 80/ 100mg/ml Sorbitol buffer. Both columns were run at 1ml/min using a 50mM NaOAc/ 150mM NaCl/ 50mg/ml sorbitol/ 0.004% Tween 80 pH 4.0 buffer.

RESULTS and DISCUSSION

1. SPHP Purification

Although a variety of SPHP purified materials were used, only one example will be presented here.

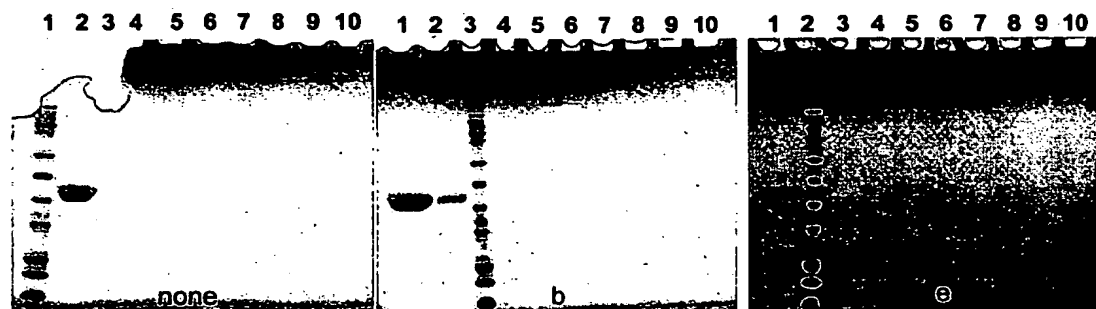
57ml of a GCSF pegylation reaction (0.35mg/ml) combined with 67ml of buffer A and diluted to 300ml with H₂O (pH 4.56, 3.5mS/cm) were loaded onto two tandem 5ml SPHP HiTrap columns pre-equilibrated with 20mM NaOAc/ 5mM NaCl pH 4.5 (A). Protein was eluted with a 30CV gradient from A to 30% of 20mM NaOAc/ 1M NaCl pH 4.5 (B) followed by a gradient from 30-100% B over 5CVs. 8ml fractions were collected. 2ml of 250mg/ml D-sorbitol and 40 μ l of 1% (w/v) Tween 80 were added to each 8ml fraction to a final concentration of 50mg/ml sorbitol and 0.004% Tween 80. (Sorbitol and Tween 80 were not added to any other SPHP purified fractions used for the study.)



	Volume (mL)	%PEG- GCSF	PEG- GCSF area	Vol*area	% GCSF	GCSF area	Vol*area	A280	A280/ 0.815 (mg/mL)	Mass (mg)
Load	300	62.1	1572421	471726300	37.9	961322	2.88E+08			
FT	300	-	0	0	-	0	0			
Wash	10	-	0	0	-	0	0	0.071	0.087	0.871
B10	10	91.5	976990	9769900	8.5	90741	907410	0.033	0.040	0.405
B9	10	89.1	10751836	107518360	0.9	102606	1026060	0.237	0.291	2.908
B8	10	97.1	15615468	156154680	0.7	110907	1109070	0.352	0.432	4.319
B7	10	93.9	9481266	94812660	1	105210	1052100	0.213	0.261	2.613
B6	10	89.3	2338334	23383340	4.4	114412	1144120		0.000	0.000
B2	10	48.9	969105	9691050	30.2	599111	5991110	0.033	0.040	0.405
B1	10	33.2	2633061	26330610	54.9	4350257	43502570	0.165	0.202	2.025
C1	10	46.1	1689527	16895270	39.2	1439674	14396740	0.082	0.101	1.006

2. HIC resin screen

SPHP purified PEG-GCSF fraction A8 was used for this HIC resin screen. 1ml PEG-GCSF samples were adjusted to 0.5M, 0.75M, and 1M NaCl. The HIC resin screen was performed as described in materials and methods.



0.5M NaCl

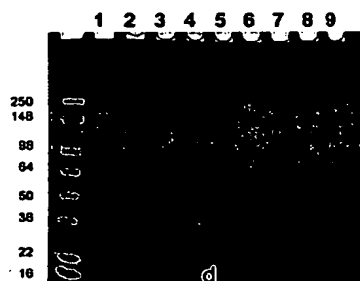
1. MW Standard
2. 0.5M NaCl Load
3. PSFF FT
4. PSFF W
5. PSFF E
6. PSFF H₂O
7. BSFF FT
8. BSFF W
9. BSFF E
10. BSFF H₂O

0.75M NaCl

1. 0.75M NaCl Load
2. PSFF FT
3. MW Standard
4. PSFF W
5. PSFF E
6. PSFF H₂O
7. BSFF FT
8. BSFF W
9. BSFF E
10. BSFF H₂O

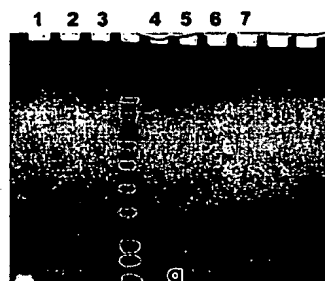
1M NaCl

1. 1M NaCl Load
2. MW Standard
3. PSFF FT
4. PSFF W
5. PSFF E
6. PSFF H₂O
7. BSFF FT
8. BSFF W
9. BSFF E
10. BSFF H₂O



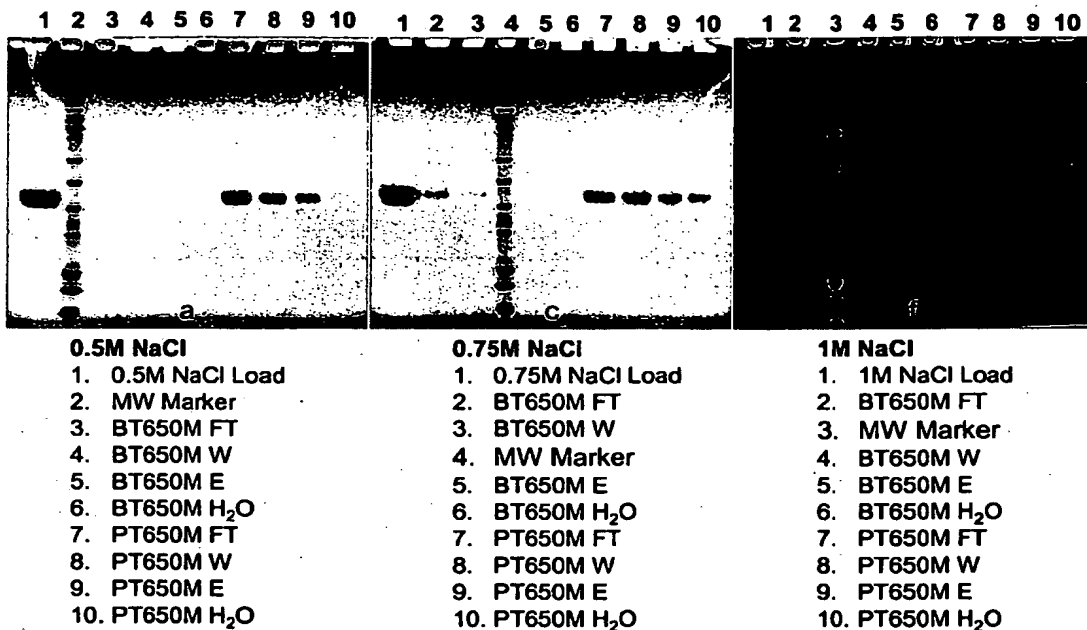
0.5M NaOH regen

1. 0.5M NaCl PSFF
2. 0.5M NaCl BSFF
3. 0.5M NaCl B650M
4. 0.5M NaCl P650M
5. 0.75M NaCl PSFF
6. 0.75M NaCl BSFF
7. 0.75M NaCl B650M
8. 0.5M NaCl P650M



SPHP/ 0.5M NaOH regen

1. B1 SPHP
2. C2 SPHP
3. C1 SPHP
4. 1M NaCl PSFF
5. 1M NaCl BSFF
6. 1M NaCl B650M
7. 1M NaCl P650M



PEG-GCSF bound irreversibly to Phenyl SFF and Butyl SFF at 0.5, 0.75 and 1M NaCl under above conditions. No PEG-GCSF was observed in flow through, wash, elutions or regeneration, except for the 0.75M NaCl PSFF flow through.

PEG-GCSF was observed in flow through and wash when applied onto Butyl 650M in 0.75M NaCl but not 0.5M NaCl. This was unexpected, as binding strength should increase with increasing salt concentration. PEG-GCSF was observed in flow through, wash, NaOAc and H₂O elution when applied onto Phenyl 650M at 0.5M and 0.75M NaCl. Comparing binding strength of these two resins, stronger binding would have been expected to the phenyl rather than butyl resin. It appears that PEG-GCSF has a greater affinity for the butyl ligand relative to the phenyl ligand.

3. HIC Purification of PEG-GCSF

3.1. Binding of PEG-GCSF to HiTrap Phenyl FF (High sub) and Phenyl Toyopearl 650M in the presence of NaCl

Chromatographic binding to 1ml HiTrap Phenyl FF (High sub) and 1ml Phenyl Toyopearl 650M in the presence of up to 500mM NaCl was investigated:

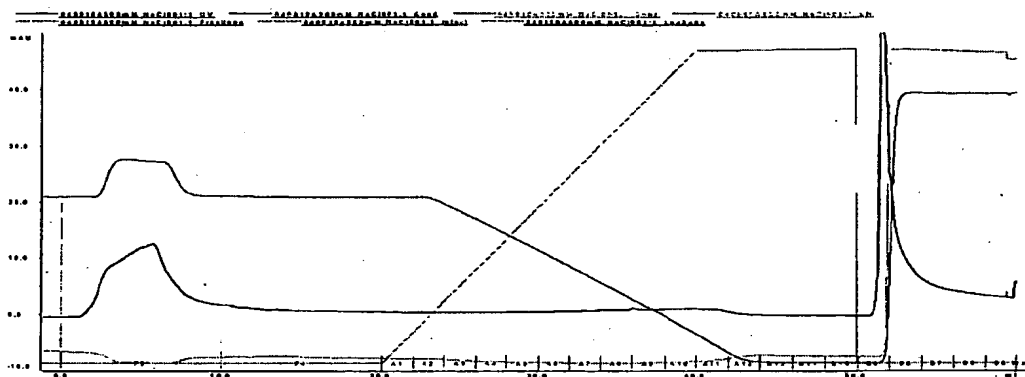
500mM NaCl – HiTrap Phenyl FF

Load: Combined A9/A12 of 040902 SPHP run
used 5mL and adjusted conductivity from 15mS/cm to 45mS/cm with ca. 550-650ul 5M NaCl

A: 10mM NaOAc/ 0.5M NaCl pH 4.5 (conductivity 45mS/cm)

B: 20mM NaOAc pH 4.5

Column: HiTrap 1ml Phenyl FF (High Sub)



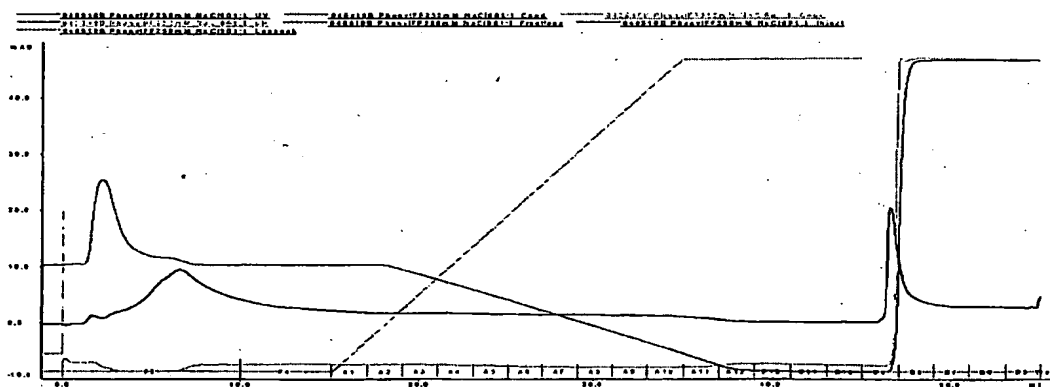
250mM NaCl – HiTrap Phenyl FF

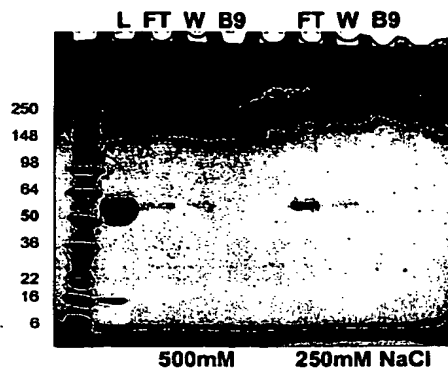
Load: 5ml A9/A12 of SPHP 040902 with NaCl added to 25mS/cm

A: 20mM NaOAc/ 250mM NaCl pH 4.5

B: 20mM NaOAc pH 4.5

Column: HiTrap Phenyl FF 1ml





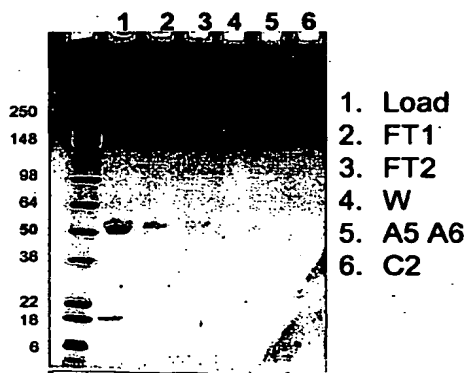
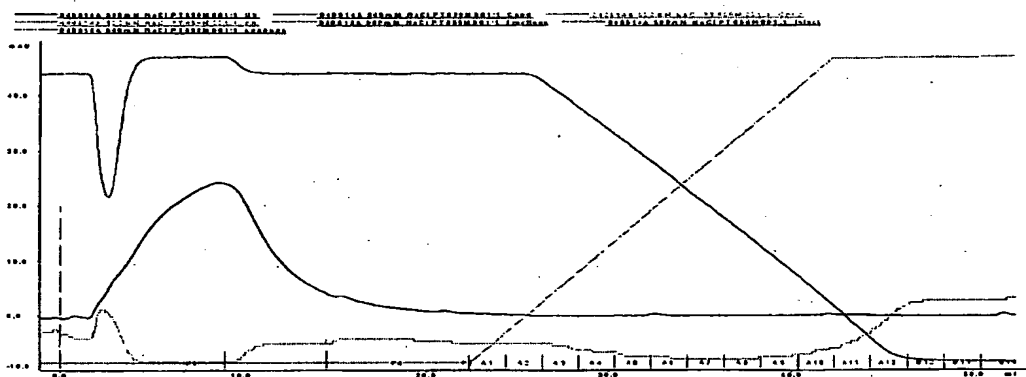
500mM NaCl – Phenyl Toyopearl 650M

3mL of 040902SPHP + 300ul 5M NaCl + 5mL buffer A

A: 10mM NaOAc/ 500mM NaCl pH 4.5

B: 20mM NaOAc pH 4.5

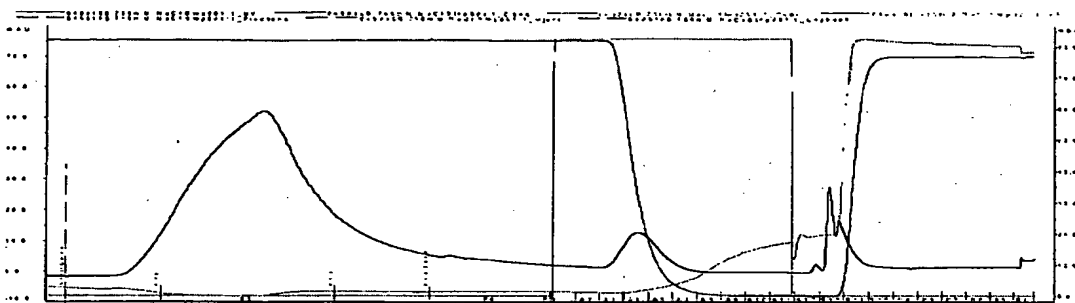
1ml Phenyl Toyopearl 650M



In both cases PEG-GCSF was observed in the flow through and wash but no protein could be eluted during gradient elution from 10 or 20 mM NaOAc/ 0.5M NaCl pH 4.5 to 20mM NaOAc pH 4.5.

NaCl concentration of the load was increased to 0.75 and 1.3M NaCl for loading onto a 1ml Toyopearl 650M column. A step elution from 20mM NaOAc/ 0.75 NaCl pH 4.5 to H₂O was performed:

0.75M NaCl Load

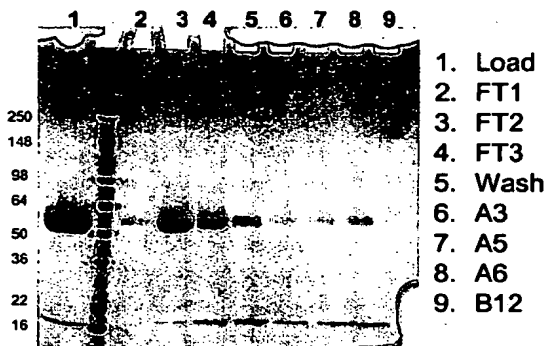


• 7mL A7/8/11 040902SPHP + 1080ul 5M NaCl

• Conductivity measured was only 40mS/cm, but suspected something is wrong with probe
• expected 80mS/cm

• A: 20mM NaOAc/ 750mM NaCl pH 4.5
• B: H₂O

• 1ml Phenyl Toyopearl 650M
• step elution

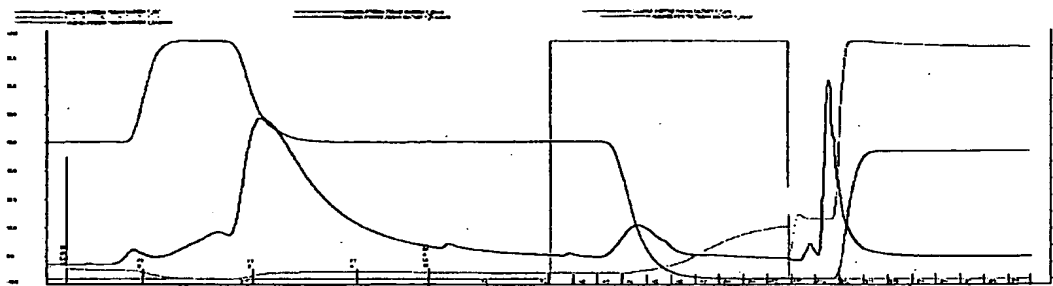


Sample #	Description	Peak Type	RT (min)	Area	% Area	Conc (mg/mL)	Volume (mL)	Mass (mg)
1	L	Aggregated Material	n/a	0	0.0	0.000		0.000
		PEG-gCSF	9.1	5154300	80.1	0.057	7	0.398
		gCSF	10.2	1277320	19.9	0.014	7	0.098
2		Aggregated Material	n/a	0	0.0	0.000		0.000
		PEG-gCSF	9.2	164087	100.0	0.002	4.4	0.008
		gCSF	n/a	0	0.0	0.000	4.4	0.000
3		Aggregated Material	n/a	0	0.0	0.000		0.000
		PEG-gCSF	n/a	0	0.0	0.000	8.2	0.000
		gCSF	10.4	10122685	100.0	0.116	8.2	0.949
4		Aggregated Material	n/a	0	0.0	0.000		0.000
		PEG-gCSF	9.2	7812910	69.2	0.088	4.4	0.387
		gCSF	10.2	3481119	30.8	0.038	4.4	0.167
5		Aggregated Material	n/a	0	0.0	0.000		0.000
		PEG-gCSF	n/a	0	0.0	0.000	4.9	0.000
		gCSF	n/a	0	0.0	0.000	4.9	0.000
6		Aggregated Material	n/a	0	0.0	0.000		0.000
		PEG-gCSF	n/a	0	0.0	0.000	1	0.000
		gCSF	10.2	215933	100.0	0.002	1	0.002
7		Aggregated Material	n/a	0	0.0	0.000		0.000
		PEG-gCSF	9.3	148291	27.1	0.002	1	0.002
		gCSF	10.2	398392	72.9	0.004	1	0.004
8		Aggregated Material	n/a	0	0.0	0.000		0.000
		PEG-gCSF	9.2	178195	18.5	0.002	1	0.002
		gCSF	10.2	787009	81.5	0.008	1	0.008
9		Aggregated Material	n/a	0	0.0	0.000		0.000
		PEG-gCSF	n/a	0	0.0	0.000	1	0.000
		gCSF	n/a	0	0.0	0.000	1	0.000

Under these conditions partial binding of PEG-GCSF to Phenyl Toyopearl 650M resin was achieved. At a 0.75M NaCl concentration in the load, most PEG-GCSF was located in the flow through and only a very small amount eluted.

Elution from 20mM NaOAc/ 1.3M NaCl pH 4.5 was performed stepwise by first eluting with 20mM NaOAc/ 0.75M NaCl pH 4.5 followed by an H₂O step elution:

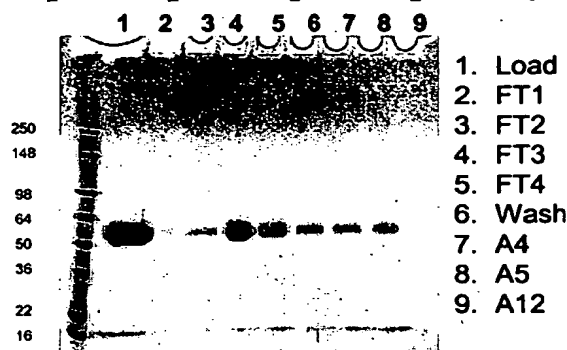
1.3M NaCl Load



• 5mL of glycopegylated GCSF + NaCl to 80mS/cm total volume ca.6.5mL (-1mL for analysis, only 5.5mL loaded)

• A: 20mM NaOAc/750mM NaCl pH 4.5
• B: H₂O

• 1ml Toyopearl Phenyl 650M
• step elution



Sample #	AC Number	Description	Peak Type	RT (min)	Area	% Area	Conc (mg/mL)	Volume (mL)	Mass (mg)
1			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	9.1	4102495	79.2	0.045	5.5	0.247
			gCSF	10.2	1078880	20.8	0.012	5.5	0.064
2			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	n/a	0	0.0	0.000	3.3	0.000
			gCSF	n/a	0	0.0	0.000	3.3	0.000
3			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	n/a	0	0.0	0.000	5.4	0.000
			gCSF	n/a	0	0.0	0.000	5.4	0.000
4			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	9.2	3059462	92.2	0.033	5.2	0.173
			gCSF	10.2	258853	7.8	0.003	5.2	0.014
5			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	9.2	970200	81.8	0.010	3.8	0.040
			gCSF	10.2	215950	18.2	0.002	3.8	0.009
6			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	9.2	284430	63.1	0.003	4.8	0.015
			gCSF	10.2	166489	36.9	0.002	4.8	0.008
7			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	9.2	137230	45.2	0.001	1	0.001
			gCSF	10.2	166583	54.8	0.002	1	0.002
8			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	9.2	312518	52.1	0.003	1	0.003
			gCSF	10.2	287424	47.9	0.003	1	0.003
9			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	9.2	401360	43.0	0.004	1	0.004
			gCSF	10.2	532938	57.0	0.006	1	0.006
10			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	n/a	0	0.0	0.000	1	0.000
			gCSF	n/a	0	0.0	0.000	1	0.000

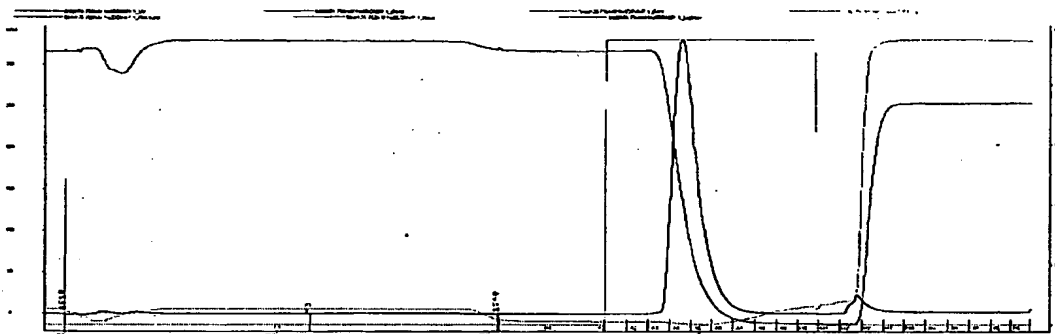
Even at 1.3M NaCl concentration in the P650M load some PEG-GCSF is located in the flow through, but most PEG-GCSF elutes during the change from 1.3 to 0.75M NaCl.

Some PEG-GCSF bound more tightly to the column and eluted during the H₂O step elution. No separation of GCSF from PEG-GCSF was achieved.

3.2. Binding of PEG-GCSF to Phenyl Toyopearl 650M in the presence of Na₂SO₄

In order to promote stronger binding, Na₂SO₄ was used instead of NaCl. A variety of Na₂SO₄ concentrations were used to find an optimal concentration range. Step elution to water was performed from 750mM Na₂SO₄/ 20mM NaOAc pH 4.1.

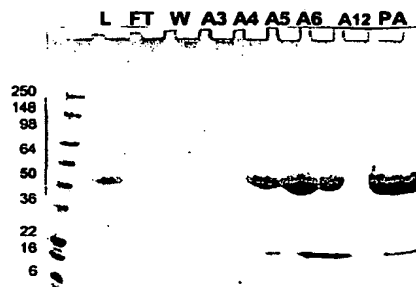
750mM Na₂SO₄ – Step Elution



- Sample: 8mL of glycopegylated GCSF + 10.4mL of 1.33M Na₂SO₄ (final ca. 750mM Na₂SO₄)

- A: 750mM Na₂SO₄/ 20mM NaOAc pH 4.1
- B: H₂O

- 1ml Phenyl Toyopearl 650M



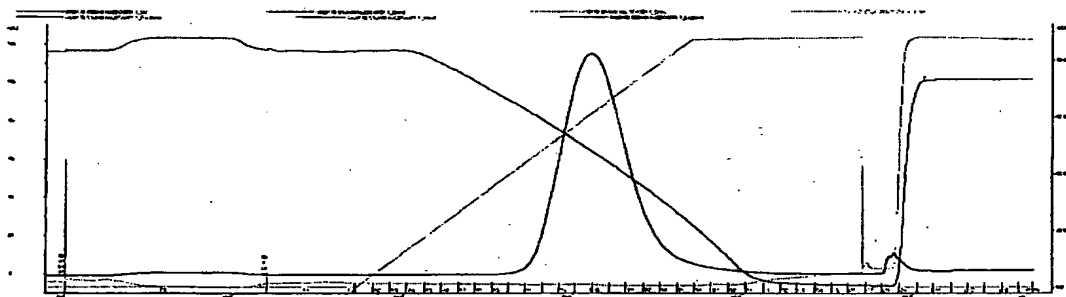
PA – Precipitate of 750mM Na₂SO₄ Load in urea, 10 and 30ul loaded

Sample #	AC Number	Description	Peak Type	RT (min)	Area	% Area	Conc (mg/mL)	Volume (mL)	Mass (mg)
1			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	9.2	1354144	72.1	0.015	17.40	0.254
			gCSF	10.2	525302	27.9	0.006	17.40	0.098
2			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	n/a	0	0.0	0.000	12.00	0.000
			gCSF	n/a	0	0.0	0.000	12.00	0.000
3			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	n/a	0	0.0	0.000	9.20	0.000
			gCSF	n/a	0	0.0	0.000	9.20	0.000
4			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	n/a	0	0.0	0.000	4.80	0.000
			gCSF	n/a	0	0.0	0.000	4.80	0.000
5			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	n/a	0	0.0	0.000	1.00	0.000
			gCSF	n/a	0	0.0	0.000	1.00	0.000
6			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	9.1	6364607	83.8	0.071	1.00	0.071
			gCSF	10.1	1231441	16.2	0.013	1.00	0.013
7			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	9.0	18410950	77.1	0.226	1.00	0.226
			gCSF	10.1	5482068	22.9	0.081	1.00	0.081
8			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	9.1	6972328	69.7	0.078	1.00	0.078
			gCSF	10.1	3032446	30.3	0.033	1.00	0.033
9			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	n/a	0	0.0	0.000	1.00	0.000
			gCSF	n/a	0	0.0	0.000	1.00	0.000

5 -8: 148% mass yield PEG -GCSF

A 20CV Gradient elution to H₂O was performed for a 650mM Na₂SO₄/ 20mM NaOAc pH 4.5 load.

650mM Na₂SO₄ – Gradient Elution

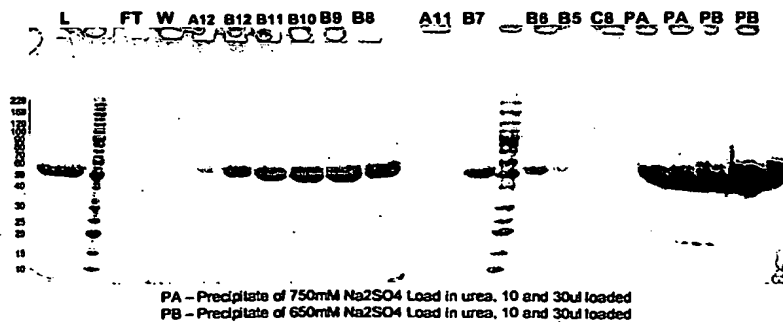


• Load: 5ml of glycopegylated GCSF
Peak1 Ref. 790 -61 + 5ml of 1.33M Na₂SO₄ (=0.665M)

• A: 0.65M Na₂SO₄/ 20mM NaOAc pH 4.5
• B: H₂O

• 1ml Phenyl Toyopearl 650M

• 20CV gradient

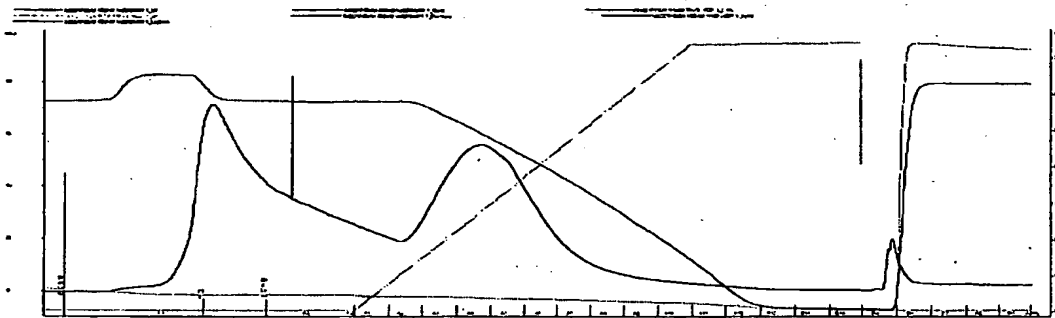


Sample #	AC Number	Description	Peak Type	RT (min)	Area	% Area	Conc (mg/mL)	Volume (mL)	Mass (mg)
1			Aggregated Material	8.1, 10.1	319212	5.2	0.003	9.00	0.031
			PEG-gCSF	9.0	5580446	91.9	0.062	9.00	0.558
			gCSF	10.4	175174	2.9	0.002	9.00	0.017
2			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	9.2	131951	57.1	0.001	14.00	0.020
			gCSF	10.5	98942	42.9	0.001	14.00	0.015
3			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	n/a	0	0.0	0.000	5.00	0.000
			gCSF	n/a	0	0.0	0.000	5.00	0.000
4			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	n/a	0	0.0	0.000	1.00	0.000
			gCSF	n/a	0	0.0	0.000	1.00	0.000
5			Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	9.2	116893	100.0	0.001	1.00	0.001
			gCSF	n/a	0	0.0	0.000	1.00	0.000
6		A12	Aggregated Material	n/a	0	0.0	0.000		0.000
			PEG-gCSF	9.3	787327	100.0	0.008	1.00	0.008
			gCSF	n/a	0	0.0	0.000	1.00	0.000
7		R12	Aggregated Material	8.5	130997	3.7	0.001	1.00	0.001
			PEG-gCSF	9.3	3438212	96.3	0.038	1.00	0.038
			gCSF	n/a	0	0.0	0.000	1.00	0.000
8		R11	Aggregated Material	8.2	306467	4.0	0.003	1.00	0.003
			PEG-gCSF	9.0	7357583	96.0	0.082	1.00	0.082
			gCSF	n/a	0	0.0	0.000	1.00	0.000
9		R10	Aggregated Material	8.1	379387	3.5	0.004	1.00	0.004
			PEG-gCSF	8.9	10351152	98.5	0.119	1.00	0.119
			gCSF	n/a	0	0.0	0.000	1.00	0.000
10		R9	Aggregated Material	8.4	522116	4.7	0.006	1.00	0.006
			PEG-gCSF	9.3	10590271	95.3	0.122	1.00	0.122
			gCSF	n/a	0	0.0	0.000	1.00	0.000
Sample #	AC Number	Description	Peak Type	RT (min)	Area	% Area	Conc (mg/mL)	Volume (mL)	Mass (mg)
1		R8	Aggregated Material	8.1	313422	3.9	0.003	1.00	0.003
			PEG-gCSF	8.0	7641418	96.1	0.086	1.00	0.086
			gCSF	n/a	0	0.0	0.000	1.00	0.000
2		R7	Aggregated Material	8.2	240761	4.8	0.003	1.00	0.003
			PEG-gCSF	9.1	4728750	95.2	0.052	1.00	0.052
			gCSF	n/a	0	0.0	0.000	1.00	0.000
3		R6	Aggregated Material	8.1	122061	5.1	0.001	1.00	0.001
			PEG-gCSF	9.1	2250804	94.9	0.024	1.00	0.024
			gCSF	n/a	0	0.0	0.000	1.00	0.000
4		R5	Aggregated Material	n/a	0	0.0	0.000	1.00	0.000
			PEG-gCSF	9.1	1099448	100.0	0.012	1.00	0.012
			gCSF	n/a	0	0.0	0.000	1.00	0.000
5			Aggregated Material	n/a	0	0.0	0.000	1.00	0.000
			PEG-gCSF	9.0	545269	100.0	0.006	1.00	0.006
			gCSF	n/a	0	0.0	0.000	1.00	0.000

A12-B5: 97.6% mass yield PEGGCSF

20CV gradient elutions were also performed from 0.4, 0.5 and 0.6M Na₂SO₄/ 20mM NaOAc pH 4.0 to 20mM NaOAc pH 4.0.

400mM Na₂SO₄ – Gradient Elution

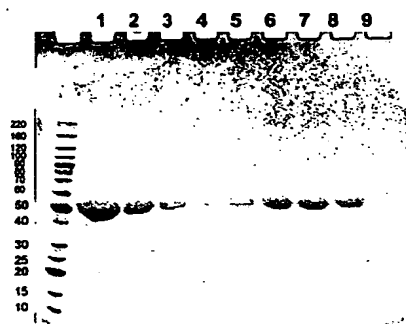


• Load: 5ml of glycopegylated GCSF
Peak1 Ref. 790 -61 + 2.15ml of 1.33M
Na₂SO₄ (=0.4M)

• A: 0.4M Na₂SO₄/ 20mM NaOAc pH 4.0
• B: 20mM NaOAc pH 4.0

• 1ml Phenyl Toyopearl 650M

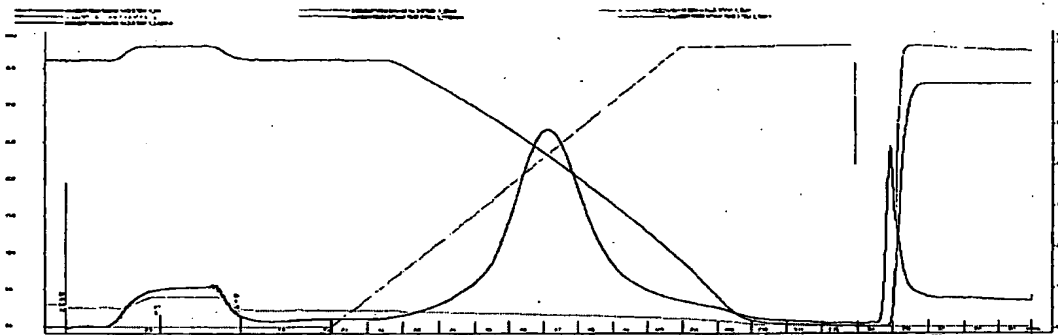
• 20CV gradient



1. Load
2. FT2
3. W
4. A2
5. A3
6. A4
7. A5
8. A6
9. A7

Sample #	AC Number	Description	Peak Type	RT (min)	Area	% Area	Conc (mg/ml)	Volume (ml)	Mass (mg)
1			Aggregated Material	8.4	1124303	8.5	0.012		0.00
			PEG-gCSF	9.3	16058036	93.5	0.183	6.10	1.18
			gCSF	n/a	0	0.0	0.000	6.10	0.00
2			Aggregated Material	n/a	0	0.0	0.000		0.00
			PEG-gCSF	n/a	0	0.0	0.000	9.80	0.00
			gCSF	n/a	0	0.0	0.000	9.80	0.00
3			Aggregated Material	8.2	303272	4.8	0.003		0.00
			PEG-gCSF	9.0	6001432	95.2	0.087	4.30	0.28
			gCSF	n/a	0	0.0	0.000	4.30	0.00
4			Aggregated Material	8.2	161261	5.0	0.002		0.00
			PEG-gCSF	9.1	3075197	95.0	0.033	5.00	0.17
			gCSF	n/a	0	0.0	0.000	5.00	0.00
5			Aggregated Material	n/a	0	0.0	0.000		0.00
			PEG-gCSF	9.1	1150548	100.0	0.012	2.00	0.02
			gCSF	n/a	0	0.0	0.000	2.00	0.00
6			Aggregated Material	n/a	0	0.0	0.000		0.00
			PEG-gCSF	9.2	1560568	100.0	0.017	2.00	0.03
			gCSF	n/a	0	0.0	0.000	2.00	0.00
7			Aggregated Material	8.2	239357	5.0	0.003		0.00
			PEG-gCSF	9.1	4595015	95.0	0.051	2.00	0.10
			gCSF	n/a	0	0.0	0.000	2.00	0.00
8			Aggregated Material	8.0	284938	4.8	0.003		0.00
			PEG-gCSF	8.9	5223758	95.2	0.058	2.00	0.12
			gCSF	n/a	0	0.0	0.000	2.00	0.00
9			Aggregated Material	8.3	314617	7.0	0.003		0.00
			PEG-gCSF	9.3	4195072	93.0	0.046	2.00	0.09
			gCSF	n/a	0	0.0	0.000	2.00	0.00
10			Aggregated Material	8.4	208499	8.2	0.002		0.00
			PEG-gCSF	9.4	2335332	91.8	0.025	2.00	0.05
			gCSF	n/a	0	0.0	0.000	2.00	0.00
11			Aggregated Material	n/a	0	0.0	0.000		0.00
			PEG-gCSF	n/a	0	0.0	0.000	2.00	0.00
			gCSF	n/a	0	0.0	0.000	2.00	0.00
12			Aggregated Material	8.3	105304	8.7	0.001		0.00
			PEG-gCSF	9.2	1111822	91.3	0.012	2.00	0.02
			gCSF	n/a	0	0.0	0.000	2.00	0.00

500mM Na₂SO₄ – Gradient Elution

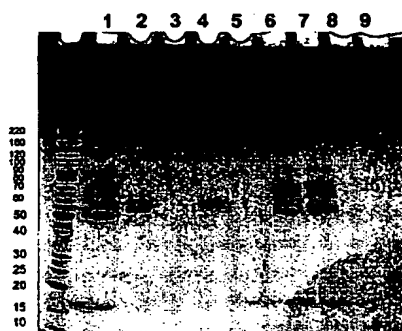


• Load: 5ml of mix of glycopegylated GCSF (ca. BB/7, 2ml B9, 9.7ml B10, 9.5ml B1) + 3.01mL of 1.33M Na₂SO₄ (-1ml for analyses, 7ml loaded)

• A: 0.5M Na₂SO₄/ 20mM NaOAc pH 4.0
• B: 20mM NaOAc pH 4.0

• 1ml Phenyl Toyopearl 650M

• 20CV gradient

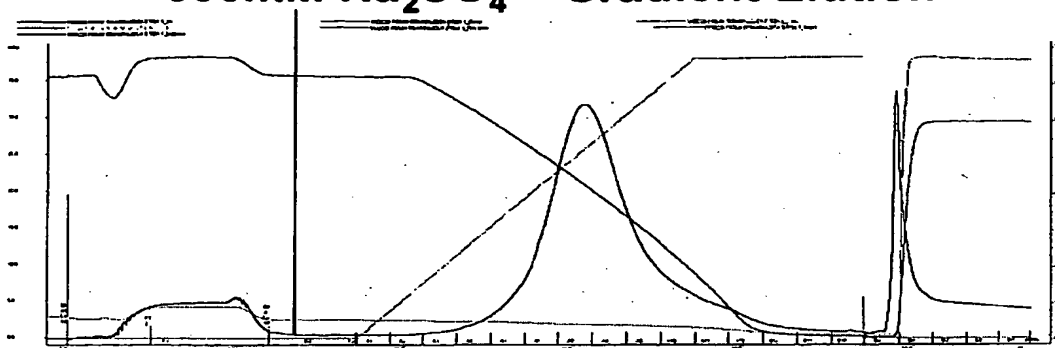


1. Load
2. FT1
3. FT2
4. W
5. A4/5
6. A6/7
7. A8/9
8. A10/11
9. B9

Sample #	AC Number	Description	Peak Type	RT (min)	Area	% Area	Conc (mg/mL)	Volume (mL)	Mass (mg)
1			Aggregated Material	8.4, 9.5	603235	19.4	0.008	7	0.045
			PEG-gCSF	9.1	1573636	50.7	0.017	7	0.119
			gCSF	9.9	926063	29.8	0.010	7	0.070
2		FT1	Aggregated Material	n/a	0	0.0	0.000	8.4	0.000
			PEG-gCSF	9.0	470015	100.0	0.005	8.4	0.032
			gCSF	n/a	0	0.0	0.000	8.4	0.000
3			Aggregated Material	n/a	0	0.0	0.000	5.4	0.000
			PEG-gCSF	n/a	0	0.0	0.000	5.4	0.000
			gCSF	n/a	0	0.0	0.000	5.4	0.000
4			Aggregated Material	n/a	0	0.0	0.000	4.8	0.000
			PEG-gCSF	9.1	230844	100.0	0.002	4.8	0.012
			gCSF	n/a	0	0.0	0.000	4.8	0.000
5			Aggregated Material	9.7	72683	29.9	0.001	4	0.003
			PEG-gCSF	n/a	0	0.0	0.000	4	0.000
			gCSF	10.0	170056	70.1	0.002	4	0.007
6		A6/7	Aggregated Material	9.7	156359	9.9	0.002	4	0.007
			PEG-gCSF	9.1	1048515	65.3	0.011	4	0.045
			gCSF	9.9	397589	24.8	0.004	4	0.017
7		A8/9	Aggregated Material	9.5	166360	13.9	0.002	4	0.007
			PEG-gCSF	9.1	764509	63.1	0.008	4	0.033
			gCSF	9.9	277866	23.0	0.003	4	0.012
8			Aggregated Material	9.4	263853	63.8	0.003	4	0.011
			PEG-gCSF	n/a	0	0.0	0.000	4	0.000
			gCSF	9.8	150893	36.4	0.002	4	0.006
9			Aggregated Material	n/a	0	0.0	0.000	2	0.000
			PEG-gCSF	n/a	0	0.0	0.000	2	0.000
			gCSF	n/a	0	0.0	0.000	2	0.000

- PEG-GCSF in FT1 (26.9% mass) and Wash (10.1% mass)
- left shoulder of elution peak nonpegylatedGCSF
- main elution peak– PEG-GCSF/nonpegylatedGCSF
- right shoulder– nonpegylatedGCSF
- aggregates track through entire elution peak
- A6-A9: 65.5% mass yield PEGGCSF

600mM Na₂SO₄ – Gradient Elution

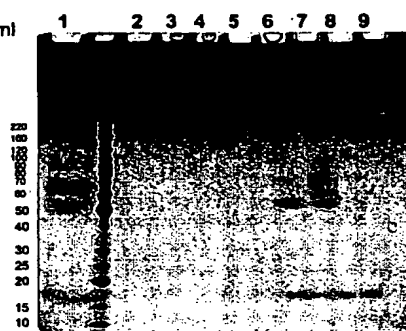


• Load: 5ml of mix of glycopegylated GCSF (ca. 2ml 2mM B9, 9.7mM B10, 9.5mM B1) + 4.11mL of 1.33M Na₂SO₄ (-1ml for analyses, 8.1ml loaded)

• A: 0.6M Na₂SO₄/ 20mM NaOAc pH 4.0
• B: 20mM NaOAc pH 4.0

• 1ml Phenyl Toyopearl 650M

• 20CV gradient



1. Load
2. FT1
3. FT2
4. W
5. A4/5
6. A6/7
7. A8/9
8. A10/11
9. B9

Sample #	AC Number	Description	Peak Type	RT (min)	Area	% Area	Conc (mg/mL)	Volume (mL)	Mass (mg)
1			Aggregated Material	8.3, 9.5	622751	22.4	0.007	8.1	0.054
			PEG-GCSF	9.1	1265203	45.6	0.014	8.1	0.113
			GCSF	10.0	884342	31.1	0.009	8.1	0.075
			n/a	n/a	0	0.0	0.000	5.9	0.000
2		FT1	Aggregated Material	n/a	0	0.0	0.000	5.9	0.000
			PEG-GCSF	n/a	0	0.0	0.000	5.9	0.000
			GCSF	n/a	0	0.0	0.000	5.9	0.000
			n/a	n/a	0	0.0	0.000	5.9	0.000
3		FT2	Aggregated Material	n/a	0	0.0	0.000	7.7	0.000
			PEG-GCSF	n/a	0	0.0	0.000	7.7	0.000
			GCSF	n/a	0	0.0	0.000	7.7	0.000
			n/a	n/a	0	0.0	0.000	7.7	0.000
4			Aggregated Material	n/a	0	0.0	0.000	4.8	0.000
			PEG-GCSF	n/a	0	0.0	0.000	4.8	0.000
			GCSF	n/a	0	0.0	0.000	4.8	0.000
			n/a	n/a	0	0.0	0.000	4.8	0.000
5		A4/5	Aggregated Material	n/a	0	0.0	0.000	4	0.000
			PEG-GCSF	n/a	0	0.0	0.000	4	0.000
			GCSF	10.1	125011	100.0	0.001	4	0.005
			n/a	n/a	0	0.0	0.000	4	0.000
6		A6/7	Aggregated Material	8.3, 9.7	244513	17.0	0.003	4	0.010
			PEG-GCSF	9.1	730825	50.7	0.009	4	0.031
			GCSF	10.0	485522	32.3	0.005	4	0.020
			n/a	n/a	0	0.0	0.000	4	0.000
7		A8/9	Aggregated Material	8.4, 9.7	335068	15.4	0.004	4	0.014
			PEG-GCSF	9.1	1350078	62.6	0.015	4	0.059
			GCSF	9.9	479138	22.0	0.005	4	0.021
			n/a	n/a	0	0.0	0.000	4	0.000
8		A10/11	Aggregated Material	8.5, 9.4	179338	44.4	0.003	4	0.008
			PEG-GCSF	9.2	94181	23.3	0.001	4	0.004
			GCSF	9.9	129864	32.2	0.001	4	0.005
			n/a	n/a	0	0.0	0.000	2	0.000
9			Aggregated Material	n/a	0	0.0	0.000	2	0.000
			PEG-GCSF	n/a	0	0.0	0.000	2	0.000
			GCSF	n/a	0	0.0	0.000	2	0.000
			n/a	n/a	0	0.0	0.000	2	0.000

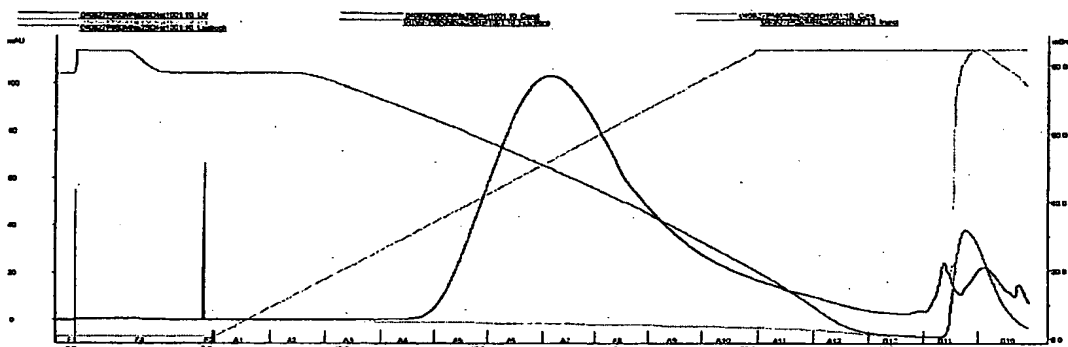
- no PEG-GCSF in FT and Wash
- left shoulder of elution peak - nonpegylated GCSF
- main elution peak - PEG-GCSF/nonpegylated GCSF
- right shoulder - nonpegylated GCSF
- aggregates track through entire elution peak
- A6-A11: 83.2% mass yield PEG-GCSF

PEG-GCSF binds to Phenyl Toyopearl 650M in the presence of Na_2SO_4 . No separation of PEG-GCSF from GCSF was achieved. Some PEG-GCSF was observed in the flow through at Na_2SO_4 concentrations of up to 500mM. Partial PEG-GCSF precipitation occurred at 650mM and 750mM Na_2SO_4 . No precipitation occurred and no PEG-GCSF was observed in the flow through at a concentration of 600mM Na_2SO_4 .

3.2.1. Confirmation of a suitable HIC purification method

The experiment was repeated at 600mM Na_2SO_4 using more representative starting material.

600mM Na_2SO_4 – Gradient Elution



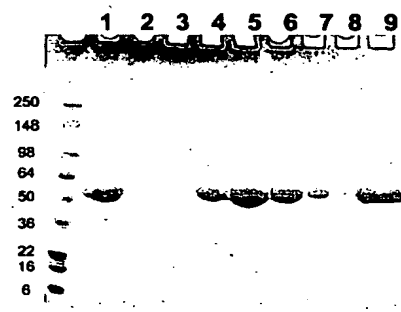
Load: 10mL glycopeglylated GCSF +8.2ml 1.33M

1ml Toyopearl 650M

A: 600mM Na_2SO_4 / 20mM NaOAc pH 4.0

B: 20mM NaOAc pH 4.0

20CV gradient



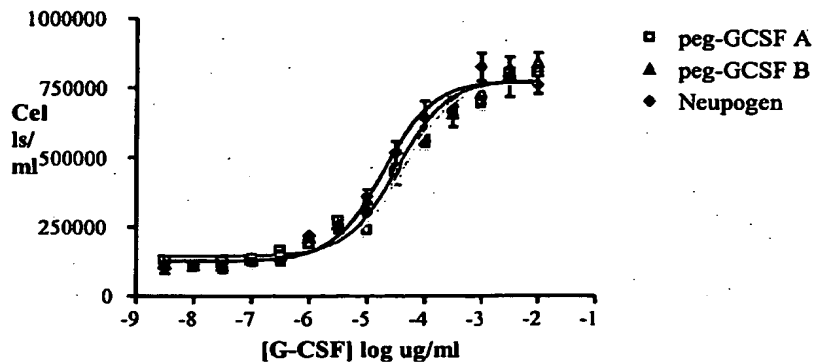
1. Load
2. FT
3. Wash
4. A5/6
5. A7/8
6. A9/10
7. A11/12
8. B12/11
9. A5-A8

Sample #	AC Number	Description	Peak Type	RT (min)	Area	% Area	Conc (mg/ml)	Volume (ml)	Mass (mg)
1			Other	7.5, 8.2	1467011	25.6	0.035	17.2	0.598
			PEG-gCSF	9.0	4266141	74.4	0.103	17.2	1.771
			gCSF	n/a	0	0.0	0.000	17.2	0.000
2			Other	n/a	0	0.0	0.000	17.2	0.000
			PEG-gCSF	n/a	0	0.0	0.000	17.2	0.000
			gCSF	n/a	0	0.0	0.000	17.2	0.000
3			Other	n/a	0	0.0	0.000	5	0.000
			PEG-gCSF	n/a	0	0.0	0.000	5	0.000
			gCSF	n/a	0	0.0	0.000	5	0.000
4		A 5/6	Other	7.4, 8.2	1237673	26.1	0.028	4	0.117
			PEG-gCSF	9.0	3495374	73.9	0.084	4	0.336
			gCSF	n/a	0	0.0	0.000	4	0.000
5		A 7/8	Other	7.5, 8.2	2735791	24.9	0.065	4	0.261
			PEG-gCSF	9.0	8260927	75.1	0.205	4	0.820
			gCSF	n/a	0	0.0	0.000	4	0.000
6		A 9/10	Other	7.4, 8.0	1275698	21.7	0.030	4	0.121
			PEG-gCSF	8.9	4599996	78.3	0.111	4	0.445
			gCSF	n/a	0	0.0	0.000	4	0.000
7		A 11/12	Other	7.9	466863	21.6	0.011	4	0.044
			PEG-gCSF	8.9	1698541	78.4	0.040	4	0.161
			gCSF	n/a	0	0.0	0.000	4	0.000
8			Other	8.2	193077	24.6	0.005	2	0.005
			PEG-gCSF	9.0	434935	55.5	0.010	2	0.020
			gCSF	10.2	156325	19.9	0.004	2	0.007
3		(with shoulders included)	Other	n/a	0	0.0	0.000	8	0.000
			PEG-gCSF	9.0	7820565	100.0	0.193	8	1.548
			gCSF	n/a	0	0.0	0.000	8	0.000
4		(with shoulders cut)	Other	7.5, 8.2	1955457	25.0	0.048	8	0.372
			PEG-gCSF	9.0	5865108	75.0	0.143	8	1.145
			gCSF	n/a	0	0.0	0.000	8	0.000

- A5-A12: 99.5% mass yield PEGGCSF
- "Other" assumed to be mainly due to low purity of GCSF (95% purity)

GCSF Proliferation Assay

G-CSF Cell Proliferation Assay



Sigmoidal dose-response	peg-GCSF A	peg-GCSF B	Neupogen
Best-fit values			
BOTTOM	144914	141887	125685
TOP	750639	753960	760952
LOGEC50	-4.66	-4.779	-4.923
EC50	2.19E-05	1.66E-05	1.19E-05
SPEC. ACT. (units/ug)	45746	60132	83752

Corrected specific activities (1 µg reported previously = 2.2 µg)

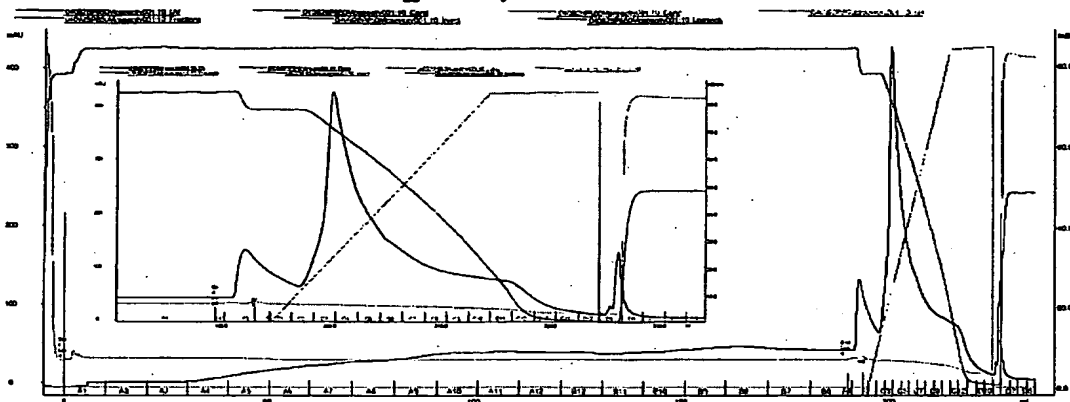
peg-GCSF A -: 20793 U/µg
peg-GCSF B: 27333 U/µg

This experiment confirmed the suitability of the HIC method – 600mM Na₂SO₄ in combination with Phenyl Toyopearl 650M resin – as a secondary PEG-GCSF purification step if needed. Precipitation was not observed and FT as well as wash did not contain any PEG-GCSF. Nonpegylated GCSF has not been found in any fractions. Tailing had not been observed during previous runs but is believed to be related to impurities (only 95% purity of GCSF used in pegylation reaction) or partial oxidation of N-terminal methionine. The mass yield of fractions A5-A12 has been determined to be 99.5%. A pool of fractions A5-A8 induced proliferation of NFS-60 cells and is therefore active.

3.2.2. PEG-GCSF binding capacity of Phenyl Toyopearl 650M

An excess of SPHP purified PEG-GCSF was applied onto a 1ml Phenyl Toyopearl 650M column to determine the binding capacity of this resin.

600mM Na₂SO₄ – Gradient Elution

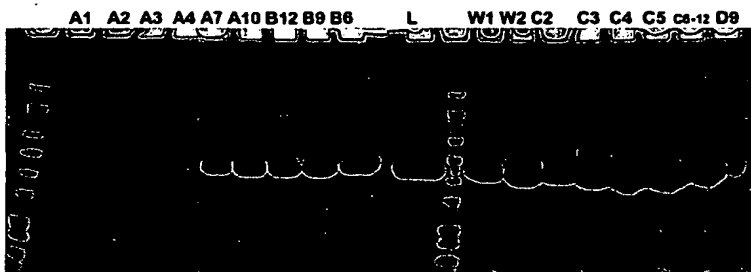


Load: 100mL of glycopegylated GCSF
+ 82mL of 1.33M Na₂SO₄ (final
600mM)

A: 20mM NaOAc/ 600mM
Na₂SO₄ pH 4
B: 20mM NaOAc pH 4

1mL Phenyl Toyopearl 650M

250
148
98
64
50
36
22
16
6



Sample #	AC Number	Description	Peak Type	RT (min)	Area	% Area	Conc (mg/mL)	Volume (mL)	Mass (mg)
1			Other	7.4, 8.1	1601241	28.0	0.038	181	6.875
			PEG-gCSF	8.9	4126099	72.0	0.099	181	18.006
			gCSF	n/a	0	0.0	0.000	181	0.000
2		A1	Other	n/a	0	0.0	0.000	10	0.000
			PEG-gCSF	n/a	0	0.0	0.000	10	0.000
			gCSF	n/a	0	0.0	0.000	10	0.000
3		A2	Other	n/a	0	0.0	0.000	10	0.000
			PEG-gCSF	n/a	0	0.0	0.000	10	0.000
			gCSF	n/a	0	0.0	0.000	10	0.000
4		A3	Other	n/a	0	0.0	0.000	10	0.000
			PEG-gCSF	n/a	0	0.0	0.000	10	0.000
			gCSF	n/a	0	0.0	0.000	10	0.000
5		A4	Other	n/a	0	0.0	0.000	10	0.000
			PEG-gCSF	9.0	262264	100.0	0.006	10	0.062
			gCSF	n/a	0	0.0	0.000	10	0.000
6		A5	Other	7.4, 8.1	380308	27.8	0.009	10	0.090
			PEG-gCSF	9.0	986648	72.2	0.023	10	0.233
			gCSF	n/a	0	0.0	0.000	10	0.000
7		A6	Other	7.4, 8.1	2880456	28.6	0.069	10	0.689
			PEG-gCSF	9.0	1520410	71.4	0.036	10	0.360
			gCSF	n/a	0	0.0	0.000	10	0.000
8		A7	Other	7.4, 8.1	786508	27.7	0.019	10	0.188
			PEG-gCSF	9.0	2077135	72.3	0.049	20	0.988
			gCSF	n/a	0	0.0	0.000	10	0.000
9		R6	Other	7.4, 8.1	1386324	27.1	0.033	10	0.328
			PEG-gCSF	9.0	3732837	72.9	0.090	10	0.898
			gCSF	n/a	0	0.0	0.000	10	0.000
10		W1	Other	7.4, 8.1	1415992	27.4	0.034	3.2	0.107
			PEG-gCSF	8.9	3754758	72.6	0.090	3.2	0.289
			gCSF	n/a	0	0.0	0.000	3.2	0.000

- no PEG-GCSF in A1-A3
- 20.6% of PEG-GCSF in A4-W2
- 24.8% of "Other" in A4W2
- capacity: 0.099mg/ml*30ml=2.97mg

Sample #	AC Number	Description	Peak Type	RT (min)	Area	% Area	Conc (mg/ml)	Volume (ml)	Mass (mg)
1		W2	Other	7.4, 8.1	3923693	28.4	0.094	1.8	0.176
			PEG-	8.9	10891383	73.5	0.276	1.8	0.498
			GCSF	n/a	0	0.0	0.000	1.8	0.000
2		C1	Other	7.4, 8.1	2787875	28.4	0.087	2	0.134
			PEG-	8.9	7656575	72.5	0.189	2	0.378
			GCSF	10.4	108771	1.0	0.003	2	0.005
3		C2	Other	7.4, 8.2	2333593	28.5	0.056	2	0.111
			PEG-	8.9	6446271	73.4	0.158	2	0.316
			GCSF	n/a	0	0.0	0.000	2	0.000
4		C3	Other	7.4, 8.1	1618402	27.7	0.038	2	0.077
			PEG-	8.9	12037204	72.3	0.307	2	0.615
			GCSF	n/a	0	0.0	0.000	2	0.000
5		C4	Other	7.4, 8.1	8988373	28.9	0.224	2	0.448
			PEG-	8.9	22156377	71.1	0.621	2	1.243
			GCSF	n/a	0	0.0	0.000	2	0.000
6		C5	Other	7.4, 8.1	7762310	27.7	0.192	2	0.384
			PEG-	8.9	20253893	72.3	0.557	2	1.114
			GCSF	n/a	0	0.0	0.000	2	0.000
7		C6	Other	7.4, 8.1	5180293	24.1	0.128	2	0.256
			PEG-	8.9	16255890	75.8	0.430	2	0.860
			GCSF	n/a	0	0.0	0.000	2	0.000
8		C7-C12	Other	7.9	3389276	24.7	0.081	12	0.976
			PEG-	8.9	10336468	75.3	0.261	12	3.126
			GCSF	n/a	0	0.0	0.000	12	0.000
9		D9	Other	7.9	428105	24.9	0.010	2	0.020
			PEG-	8.9	1284771	75.1	0.030	2	0.061
			GCSF	n/a	0	0.0	0.000	2	0.000

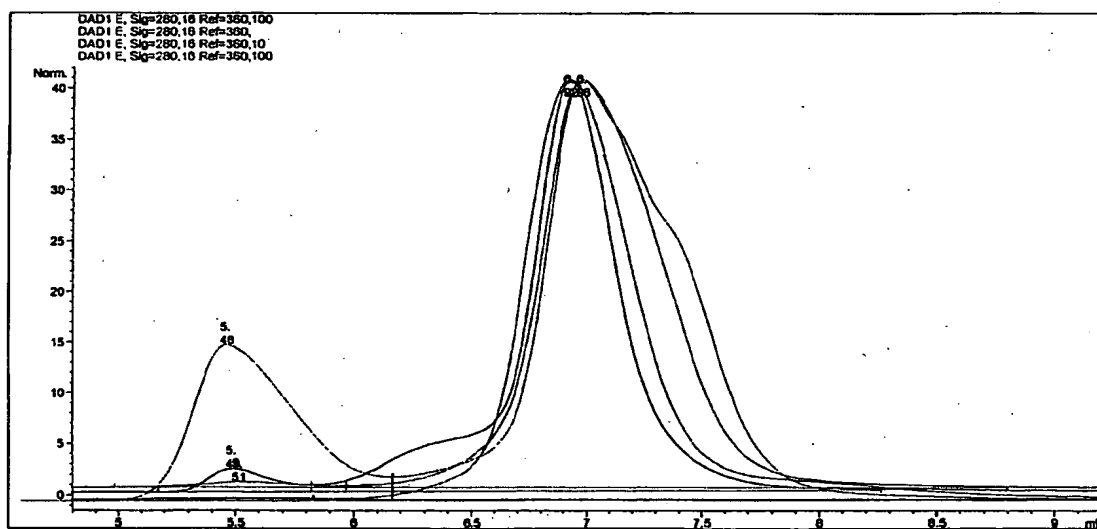
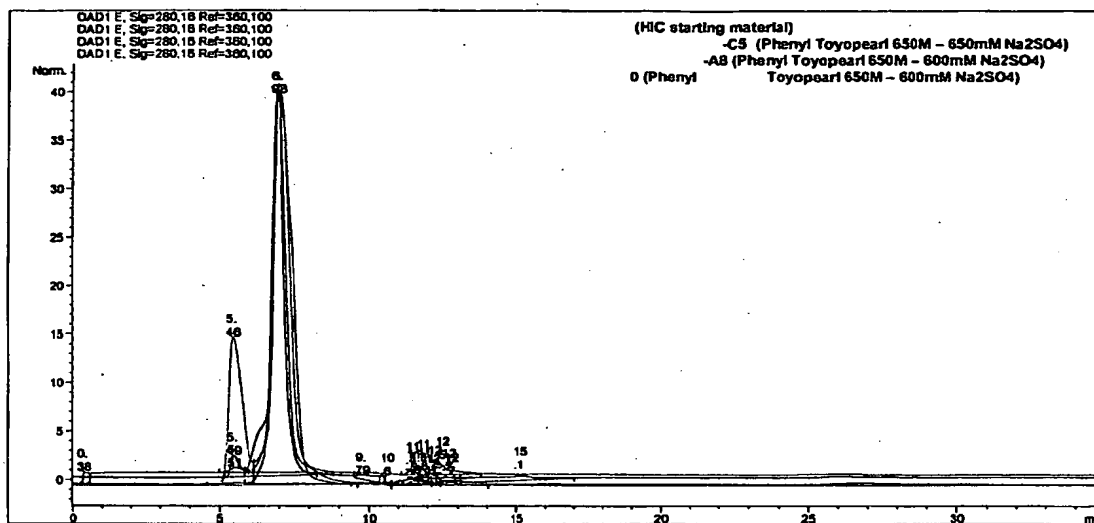
- 23.0% mass yield (4.148mg) PEGGCSF in C2-C6
- 17.3% mass yield (3.126mg) PEGGCSF in C7-12
- 40.4% mass yield (7.274mg) PEGGCSF in C2-C12
- 18.5% mass yield (1.272mg) of "others" in C2C6
- 14.2% mass yield (0.976mg) of "others" in C7C12
- 32.7% mass yield (2.248mg) of "others" in C2C12

The binding capacity for PEG-GCSF was calculated to be approximately 3mg per 1 ml of Phenyl Toyopearl 650M resin. The sample used for the capacity study contained other contaminants due to low purity of GCSF starting material. Therefore, the loading capacity may be higher if pure PEG-GCSF were used as the feedstock.

4. SEC

SEC analysis of HIC purified samples was performed to check for aggregation. Samples were run at 1ml/min in 50mM NaOAc/ 150mM NaCl/ 50mg/ml sorbitol/ 0.004% Tween 80 pH 4.0 on a G3000SW_{XL} (Tosoh Biosciences) and a Shodex OH pak (Phenomenex) column.

4.1. SEC analyses on G3000SW_{XL}



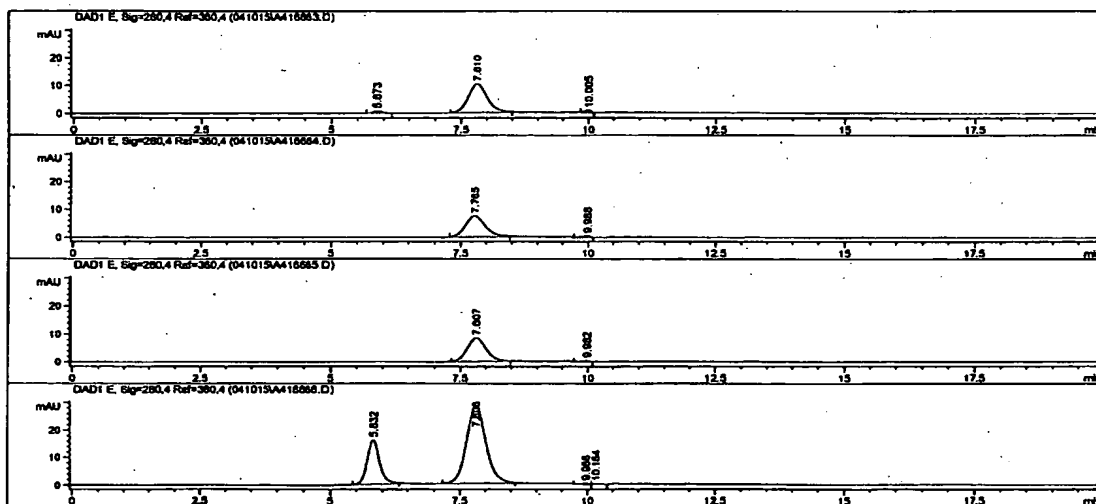
Run	Area %			
	RT 5.5min	RT <6.5min	RT 6.9min	RT η12min
1	3.36	11.88	84.75	
A12-C5			98.53	1.47
A5-A8	0.86		95.61	3.53
C4	21.03		78.12	0.85

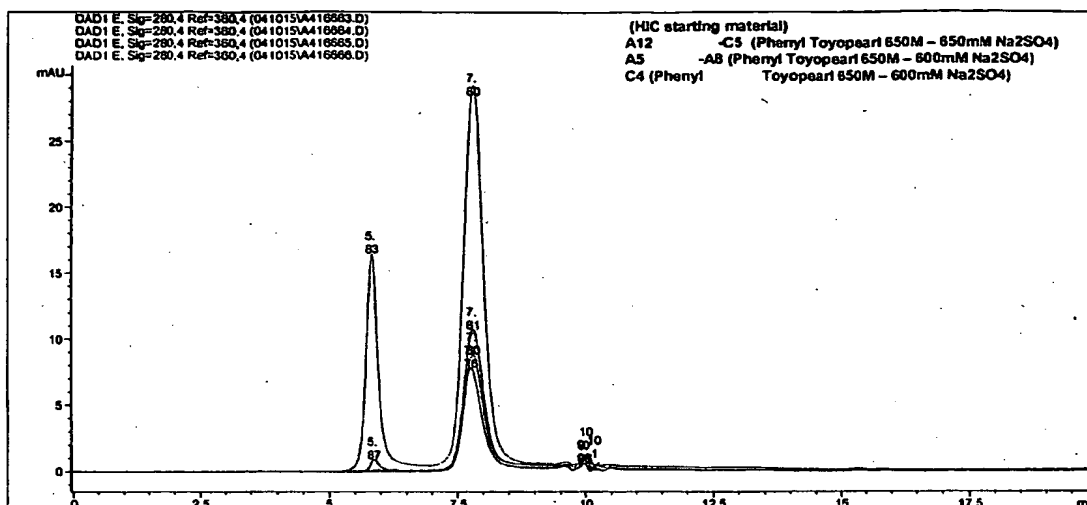
RP-HPLC data:

AC Number	Description	Peak Type	RT (min)	Area	% Area	Conc (mg/mL)	Volume (mL)	Mass (mg)
-	P8Cm	Other	8.2	2768023	24.8	0.030		
-	SPHPpoolKK	PEG-gCSF	9.0	8376291	75.2	0.085		
-	A12-B5	PEG-gCSF	9.0	5835941	100.0	0.142		
-	A5-A8	PEG-gCSF	9	7820585	100	0.189	8	1.54
-	C4	Other	7.4, 8.1	8988373	28.9	0.224	2	0.449
-		PEG-gCSF	8.9	2215837	71.1	0.82	2	1.24

The 5.4min SEC peak has been identified as aggregate. Aggregation below 1% has been observed for 040917B A12-C5 and 040927 A5-A8 samples. 3.4% aggregation has been observed for a 040923SPHP KK sample, which had been used as starting material for the 040927 and 040928 (capacity study) HIC purifications. This material was a pool of SPHP PEG-GCSF fractions in a 50mM NaOAc buffer containing an unknown concentration of NaCl. 21% aggregation was observed for a 040928 C4 sample; this from an intentionally overloaded column as part of a capacity determination experiment. This aggregation was also apparent in a nonreduced 4-20% Tris-glycine gel (see gel in section 3.2.2.). It is noted that PEG GCSF eluted in the breakthrough fractions, and that aggregate was retained until the gradient elution. This suggests that more development of this HIC step may result in a method to resolve aggregate from product.

4.2. SEC analyses on Shodex OHpak column





QC #	Sample description	Peak Area, Aggregate	Peak area, PEG-GCSF	%aggregate / (aggregate+main peak)	%main peak
AC04-16663	PEG-GCSF 040923SFHP KK M	11.07	299.36	3.70	96.30
AC04-16664	PEG-gCSF HIC 040917B A12-B5	0.00	230.98	0.00	100.00
AC04-16665	PEG-gCSF HIC 040927 A5-A8	1.72	245.73	0.70	99.30
AC04-16666	PEG-gCSF HIC 040928 C4	295.56	825.99	35.78	64.22

SEC data obtained from a Shodex OHpak column show a similar trend as data obtained using a G300SW_{XL} column. Below 1% aggregation was observed for A12-C5 and A5-A8 samples. 3.7% aggregation was observed for a KK sample and 35.8% for a 040928 C4 sample.

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